Biochemical Pharmacology, Vol. 34, No. 19, pp. 3399–3403, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
Pergamon Press Ltd.

COMMENTARY

ENDOGENOUS LIGANDS FOR BENZODIAZEPINE RECOGNITION SITES

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The report by Young *et al.* [1] that the putative neurotransmitter glycine participates in the action of benzodiazepines appeared at the same time in which our laboratory was obtaining strong biochemical evidence suggesting that the benzodiazepines facilitate the action of γ-aminobutyric acid (GABA) at specific synaptic receptors [2–6]. This evidence, showing a GABAergic participation in the action of benzodiazepines, cast new light on an earlier report by Schmidt *et al.* [7] that diazepam selectively enhances the presynaptic inhibition of spinal motor neurons in the cat. When Schmidt *et al.* [7] reported this finding it had not yet been established that GABA participates in the presynaptic inhibition of motor neurons; therefore, the enhancement of presynaptic inhibition by the benzodiazepines could not be interpreted in terms of an involvement of GABA. The implication that GABA participates in the presynaptic inhibition induced by benzodiazepines was advanced by Polc *et al.* [8] who, while confirming the findings of Schmidt *et al.* [7], showed that in the spinal cord the enhancement of presynaptic inhibition by benzodiazepines could be antagonized reversibly by bicuculline, a specific GABA receptor antagonist. Moreover, Polc *et al.* [8] and Banna *et al.* [9] reported that benzodiazepines lose their facilitatory effect on the presynaptic inhibition when GABA synthesis is blocked, thereby implying that GABA was necessary for the expression of benzodiazepine action. A similar GABA/benzodiazepine interaction was found to be operative in the facilitation of the presynaptic inhibition by benzodiazepines in many other brain areas [10, 11], thereby leading to the generalization that the benzodiazepines act by facilitating GABAergic transmission [10, 11]. That the benzodiazepines bind with high affinity to a specific synaptic site became clear from independent reports by Squires and Braestrup [12] and Mohler and Okada [13]. This evidence triggered several lines of independent investigations, each reflecting a different research strategy and philosophy taken by various laboratories.

GABA/Benzodiazepine interactions

At a meeting of the British Society of Psychopharmacology held in London in April 1978, we reported [14] that benzodiazepines, added *in vitro* to crude synaptic membranes prepared from rat brain, increased the B_{max} of GABA recognition sites. It was already known [15] that crude synaptic membranes contain two populations of GABA recognition sites; one has a high affinity (K_d of about 22 nM) and the other a low affinity (K_d of about 160 nM) for GABA. We proposed that these two sites expressed two conformational states of the GABA receptors and that the expression of these states was regulated by an endogenous polypeptide (molecular weight around 10,000 daltons) which was thermostable [14, 16–18]. This polypeptide appears to compete with diazepam for its high-affinity binding site and to function as a regulator of the affinity characteristics of the GABA recognition sites; when the endogenous peptide interacts with its binding site (presumably linked to the one that binds benzodiazepines), it reduces the number of GABA recognition sites which have high affinity for GABA. Benzodiazepines compete with this peptide and weaken its capability to down-regulate the number of high-affinity GABA recognition sites [16–18]. When this regulatory site of GABA receptors, presumably located in the vicinity of the GABA recognition sites, is occupied by diazepam, the number of high-affinity recognition sites for GABA is enhanced. At that time, we proposed that drugs that mimic the action of the endogenous polypeptide may represent a new class of CNS stimulants [18]. The prediction that benzodiazepine recognition sites may recognize ligands with pharmacological action opposite to that expressed by diazepam was verified later by Braestrup and his collaborators [19] when they reported that beta-carboline-3-carboxylate (β-CCE) possesses high affinity for the benzodiazepine recognition site and that its metabolically stable derivatives cause convulsions in rodents [20] and anxiety in humans [21]. In light of these results and based on our original proposal [18], we inferred that multiple chemical signals participate in GABAergic synaptic transmission [22, 23]. This inference was considered by others an unlikely probability [24] because it contravened the then popular belief that synaptic transmission was transacted by a single chemical signal. The view that benzodiazepines were acting on a specific recognition site, physiologically coupled with GABA recognition sites, acquired greater credibility when Tallman *et al.* [25] reported that GABA

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increases the affinity of benzodiazepine recognition sites for their ligands. That the specific glycoproteins that form the benzodiazepine recognition site are part of the supramolecular structure known as the GABA receptor [26, 27] was supported by an elegant experiment conceived by Möhler and colleagues [28]. They incubated [^{3}H]flunitrazepam with brain slices in the presence of ultraviolet light and found that this benzodiazepine binds covalently to a number of specific recognition sites. In an electron microscopic study of cerebellar slices, and combining photoaffinity labeling with the specific immunochemical staining of glutamic acid decarboxylase (GAD), a specific marker for GABA neurons, Möhler and colleagues demonstrated that the covalently bound [^{3}H]flunitrazepam is consistently located *vis à vis* to nerve terminals stained with the specific marker for GABA neurons.

Endocoids for benzodiazepine recognition sites

While this research was unfolding, a number of laboratories followed alternative research strategies directed to the identification of a specific putative neurotransmitter acting on the benzodiazepine recognition sites and stored in a non-GABAergic neuronal pathway. Möhler *et al.* [29] looked for such endogenous ligands of benzodiazepine recognition sites and reported that from a brain extract purified by gel chromatography they could separate three peaks that displaced [^{3}H]diazepam from specific binding sites. Tentatively, they identified each peak as containing inosine, hypoxanthine and nicotanamide respectively. A study of these three compounds in the whole animal suggested that nicotinamide may have a profile similar to that of the endogenous ligand for benzodiazepine recognition sites [29]. This trend of looking for the transmitter that acts on the benzodiazepine recognition site was pursued by other investigators including Slater and Longman [30], Skolnick *et al.* [31, 32], Marangos *et al.* [33], and Asano and Spector [34]. These efforts did not lead to any major breakthroughs. One of the major by-products of this research trend, however, was the discovery that caffeine, an inhibitor of adenosine receptors, antagonizes many of the actions elicited by benzodiazepines. Today this antagonism is considered to express a physiological antagonism due to the CNS stimulatory action of caffeine. This is currently believed to be related to inhibition of adenosine receptors [35]. Hence, caffeine, by attenuating a physiological inhibitory mechanism, stimulates the CNS activities depressed by benzodiazepines. The doses of caffeine that were found to modify the actions of benzodiazepines were so small that they appeared to be at variance with the low affinity of caffeine for benzodiazepine recognition sites. Because of this, it could not be proposed that the inhibition of benzodiazepine action by caffeine was mediated via occupation of benzodiazepine recognition sites. Thus, an involvement of adenosine receptors in benzodiazepine action has lost credibility and, in fact, its impact on the search for the endocoid of benzodiazepine recognition sites is almost exhausted.

GABA-modulin and diazepam binding inhibitor (DBI)—Two polypeptides involved in GABAergic transmission

Further work clarified that GABAergic synapses contain at least two polypeptides involved in the modulation of GABA recognition sites. One of them is a membrane peptide [36, 37] while the other is not located in membranes [38]. Both polypeptides were purified to homogeneity [36, 38]; one of them, a protein termed GABA-modulin [39], was found to be located in postsynaptic membranes [37] and to be operative in modulating the shift in abundance of high- and low-affinity recognition sites for GABA [18, 36]. The modulatory function and potency of GABA-modulin depend on the phosphorylation of some specific sites of this polypeptide. These sites are phosphorylated by cyclic AMP-dependent protein kinase, whereas the phosphorylation of other sites by Ca^{2+}-dependent C kinase fails to modify the action of GABA-modulin on GABA recognition sites [40]. When the amino acid composition of this protein was studied it was found that it resembles that of the small molecular weight myelin basic protein. However, GABA-modulin can be differentiated from this protein by several criteria, including the polypeptide fragmentation pattern elicited by partial proteolysis [37] and immunochemistry. The other peptide that appears to participate in GABAergic transmission was called DBI (diazepam binding inhibitor) because it can displace competitively specific ligands from the benzodiazepine recognition sites. When tested in a behavioral model of anxiety in rats, DBI was shown to elicit a proconflict action when injected intraventricularly [38] and, using a radioimmunoassay, to have a specific distribution pattern within various brain nuclei (Alho *et al.*, *Science*, in press (1985)). Using trypsinization of DBI, it was shown that its proconflict action resides in a characteristic octodeca-neuropeptide (ODN) (Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys) [41] which, when injected intraventricularly, mimics the proconflict action of DBI. The proconflict actions of both DBI and ODN appear to involve the benzodiazepine recognition site because they are antagonized by RO 15-1788. This imidobenzodiazepine derivative binds to the benzodiazepine recognition sites with high affinity [42] and antagonizes the pro- and anticonflict actions of the benzodiazepine recognition site ligands [43]. Since RO 15-1788 is a benzodiazepine recognition site ligand with little intrinsic activity [42], it is considered a specific antagonist.

The amino acid sequence analysis of DBI after CNBr fragmentation suggests that DBI contains two replicas of ODN. Both DBI [38] and ODN [41] appear to displace various ligands that bind to benzodiazepine recognition sites and to have some degree of selectivity for displacing competitively various β-carboline-3-carboxylate derivatives. These compounds elicit proconflict responses and facilitate the onset of convulsion. Immunohistochemically, DBI was shown to be located intraneurally and to be present in the highest concentrations in the hypothalamic arcuate nucleus followed in decreasing order of concentrations by the ventral medial hypothalamic nucleus, supraoptic nucleus, amygadoid

nuclei, cerebellar cortex, hippocampus, and striatum. Using high concentrations of antiserum, DBI immunoreactivity is also present in glial cells and fibers, but even in these cells it is not present uniformily throughout the brain. It must be said that, since DBI is a 105 amino acid peptide, it may be the precursor of ODN and perhaps of other functionally active small peptide fragments. Hence, DBI could be the precursor of a neuropeptide functioning in neurons and of another peptide functioning in glial cells. Alternatively, the same neuropeptide may be important for glial and neuronal function. Since in glial cells DBI immunoreactivity is not detectable with low concentrations of antiserum which demonstrate DBI in neurons, one cannot exclude that the material present in glial cells is non-specific staining.

A DBI-like material was detected in human brain. This peptide cross-reacts (1/100) with an antiserum raised against rat DBI, and the specific antiserum against human DBI cross-reacts with rat DBI (also about 1/100); the amino acid compositions of the two DBIs are quite similar. The peptides generated by the tryptic digestion of the DBI prepared from the brains of the two species are also similar suggesting that the difference between the two peptides resides in the sequence of immunogenic epitopes located in proximity to the amino terminus site, which in our preparation is blocked. Since ODN was generated by tryptic digestion, we are presently working on hypothalamic extracts and are using a specific antiserum against ODN combined with HPLC to elucidate whether an ODN-like material is endogenously produced from DBI. DBI can also be detected and measured in human spinal fluid. We have synthesized ODN and found it to possess biological activity *in vivo* (proconflict action) and *in vitro* (displacement of benzodiazepine recognition site ligands) identical to that of ODN obtained by tryptic digestion of DBI [41]. Moreover, since the HPLC elution profile of synthetic ODN is identical to that of the ODN produced from DBI by tryptic digestion, the amino acid sequence of the latter is confirmed. The displacement of benzodiazepine recognition site ligands by ODN is being studied, using primary cultures of cerebellar granule cells, by adding the ^{3}H-ligand to the cell culture medium. With this technology, the ODN displaces [^{3}H] β-carbolines competitively with an IC_{50} of about 4 μM. We have synthesized the amide of the carboxyl terminal lysine of ODN and have found that it is completely inactive. We have synthesized and tested a number of ODN fragments; the octapeptide (ON), Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys, is active and deserves attention as a putative endocoid of the benzodiazepine recognition site.

Benzodiazepine–GABA interaction at the Cl^- *channel*

Several laboratories have reported that the addition of GABA activates Cl^- channels causing a flux of Cl^- to occur across neuronal membranes according to the concentration gradient [44]. Using the patch clamp technique [45], this activation occurs in bursts of repeated openings of these anionic channels [46, 47]. In the absence of GABA, the addition of benzodiazepine to neuronal membrane preparations fails to modify the kinetics of the Cl^- channels [48]. However, when benzodiazepines are added in the presence of GABA, the burst duration of Cl^- channel openings is increased [48]. While benzodiazepines fail to modify *per se* the characteristics of the single event, they prolong the burst duration elicited by GABA, thereby amplifying the efficiency of the Cl^- fluxes through specific channel openings elicited by GABA [49, 50]. Assuming that this GABA–benzodiazepine interaction reflects a physiological modulation operative in GABAergic synaptic transmission, one might speculate that there may be two types of signals participating in GABAergic synaptic transmission. These two types of signals could be termed transmitter or mediator (GABA) and cotransmitter or modulator (the DBI-derived endocoid). The term mediator refers to a signal that acts on specific recognition sites that are coupled to a transducer system. Occupation of the mediator recognition site by the appropriate ligand activates the transducer, thereby generating a stimulus for the postsynaptic cell. In the case of GABA, it would elicit a flux of Cl^- across the neuronal membrane. The term modulator refers to a synaptic signal acting on a recognition site which is coupled either to a recognition site for the mediator or to the device coupling the mediator receptor to the transducer. Hence, as exemplified by the benzodiazepines, the modulator signal is devoid of action in the absence of the mediator signal, and in fact, the modulator signal amplifies or reduces the action of the mediator signal on its specific receptor. Mediators and modulators may coexist in the same axon or may reach the same synapses after being released from two distinct axons. The possibility that in some cases the modulator is released from specialized glial cells could even be considered. The latter two possibilities appear particularly appealing because such a convergent modulation of a synapse brings information from two different neuronal fields. It appears that DBI applied to primary neuronal cultures in micromolar quantities causes no action on the Cl^- ionophore but it reduces the duration of Cl^- channel opening when applied together with GABA [51]. This channel action of DBI, like that on behavior, is reminiscent of that of β-carboline-3-carboxylates [52]. This down-regulation of GABA action elicited by DBI can be antagonized with RO 15-1788. Further work is needed to describe this DBI action properly and to understand the mechanisms that are operative in mediating this action on GABA-regulated Cl^- channels. It is clear, however, that GABA–Cl^- channel interaction can be down-regulated by endogenous peptides related to DBI by an action on receptors which is blocked by RO 15-1788, the antagonist ligand of benzodiazepine recognition sites.

Conclusion

A neuropeptide generated from DBI, a 105 amino acid brain polypeptide, appears to be a candidate for the role of endocoid of the central benzodiazepine recognition site. DBI is present in the brain of various mammals, including humans. Moreover, DBI-like immunoreactivity was detected in human spinal fluid.

This neuropeptide appears to act as a modulator in the activation of Cl^- ionophores associated with stimulation of GABA receptors. ODN, an octadecaneuropeptide that is produced by trypsinization of DBI, fulfills some of the requirements for a putative brain endocoid of benzodiazepine recognition sites; it may act as a modulator of GABA receptor by shortening the bursts of Cl^- channel opening elicited by GABA. Since derivatives of β-carboline-3-carboxylate endowed with anxiogenic activity also decrease the transmembrane Cl^- fluxes elicited by GABA [52], one can infer that ODN or a closely related congener may be a brain benzodiazepine recognition-site endocoid. By inference from behavioral experiments in rats [41], ODN may be involved in the modulation of behavior elaborated in response to stress. Hence, fear, aggression and anxiety may be related to a modulation of GABA-ergic transmission by a DBI product acting as a GABA synapse modulator. In support of this possibility is the finding that ODN injected intraventricularly facilitates convulsion and elicits pro-conflict action in rats [41]. Moreover, added to primary cultures of granule cells from cerebellum it displaces preferentially β-carbolines among the ligands that bind to benzodiazepine recognition sites. Also the ON octapeptide, a fragment of ODN, appears to have a profile similar to that of ODN.

On the basis of the present data we conclude that the endocoid of benzodiazepine recognition sites could be either ON or another closely related fragment of ODN; its physiological action could be the regulation of the onset of anxiety or fear when an individual operates in a conflict situation. It might well be that DBI or ODN (or an ODN metabolite) possess a biphasical ability to bind with different affinities to the β-carboline and to the benzodiazepine recognition site. In primary cultures of cerebellar granule cells, ODN displaces β-carbolines and benzodiazepines with different affinities. In fact, experimental data allow one to infer that these two sites may not be absolutely identical. Thus, ODN that displaces preferentially β-carbolines over anxiolytic benzodiazepine may be anxiogenic at low doses and anxiolytic at high doses, or it might well be that another peptide generated from DBI exerts a preferential anxiolytic action. Be that as it may, we consider that the neurobiology of anxiety, phobias and aggression has entered an exciting new phase, and that a concrete basis for concerted studies of the neurochemical correlates of these states has been established.

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